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Phylogenetic divergence of three morphologically similar truffles: *Tuber sphaerosporum*, *T. sinosphaerosporum*, and *T. pseudosphaerosporum* sp. nov.

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Abstract — We describe and illustrate *T. pseudosphaerosporum*, a new species from Yunnan, China. This is most closely related to the European *T. borchii* and the North American *T. gibbosum*. ITS-rDNA sequence analysis reveals the phylogenetic divergence of three morphologically similar truffle species: *T. sphaerosporum* from North America, and *T. sinosphaerosporum* and *T. pseudosphaerosporum* from China. Phylogenetically, *T. sphaerosporum* is most closely related to another North American species, *T. californicum*, while *T. sinosphaerosporum* clusters with a European species, *T. dryophilum*.

Key words — *Ascomycota*, white truffle

Introduction

In November 2012, during our yearly investigation of *Tuber* species in Yunnan Province, China, we found some white truffles in a stand of the conifer *Pinus armandii* Franch. This truffle differed from most known species but was morphologically almost indistinguishable from the North American *T. sphaerosporum* Gilkey (Gilkey 1954) and the Chinese *T. sinosphaerosporum* L. Fan et al. (Fan et al. 2013). Our aim was to resolve the phylogenetic relationships of these three species based on ITS-nrDNA sequences and to describe the new species. Our phylogenetic analysis indicates that the new species is distant from both *T. sphaerosporum* and *T. sinosphaerosporum*.

Materials & methods

Morphological studies

Fresh specimens were collected from Yunnan Province, China, and deposited in the Herbarium of the Biology Department, Capital Normal University (BJTC), Beijing.

Table 1. *Tuber* specimens and sequences used in phylogenetic analysis.

Species	Voucher	Origin	GenBank No. (ITS)
T. aestivum	TaeW064S-W140 (UPS)	Sweden	AJ888062
	TaeW028I-E157 (UPS)	Italy	AJ888118
T. borchii	GB45	Italy	HM485344
T. sphaerospermum	AH39190	Spain	JN392246
T. californicum	JT22590	USA	HM485351
	JT28058	USA	HM485346
T. dryophilum	GB69	Italy	HM485353
	GB37	Italy	HM485354
T. gibbosum	JT2789	USA	FJ809869
T. lijiangense	HKAS 52005 (Holotype)	China	KF805727
	HKAS 52005	China	GQ217541
T. microsphaerosporum	BJTC FAN152 (Holotype)	China	KF805726
T. oligospermum	MA: Fungi: 41010A	Spain	FM205506
	MA: Fungi: 41010B	Spain	FM205507
T. oregonense	JT8767	USA	FJ809879
T. pseudosphaerosporum	**BJTC FAN250 (Holotype)**	**China**	**KF744063**
	BJTC FAN260	**China**	**KF744062**
T. puberulum	TL3857	Denmark	AJ969625
T. sinosphaerosporum	BJTC FAN135 (Holotype)	China	JX092086
	BJTC FAN136	China	JX092087
T. sphaerosporum	JT12487	USA	FJ809853
	JT19772	USA	FJ809854

Macroscopic characters were described from both fresh and rehydrated specimens. Microscopic characters were described from razor-blade sections mounted in 3% KOH, Melzer's reagent, or 0.1% (w/v) cotton blue in lactic acid. For scanning electron microscopy (SEM), spores were scraped from the dried gleba, placed onto double-sided tape, mounted directly onto an SEM stub, coated with palladium, and then examined and photographed using a HITACHI S-4800 SEM.

Molecular methods

Herbarium samples were crushed by shaking for 3 min at 30 Hz (Mixer Mill MM 301, Retsch, Haan, Germany) in a 1.5 ml tube, together with one 3 mm diameter tungsten carbide ball. Total genomic DNA was then extracted using a EZNA_Fungal DNA kit (Omega Bio-Tek Inc. Norcross, Georgia, United States) following the manufacturer's protocol. The ITS region was amplified by PCR using the primers ITS1 and ITS4 (White et al. 1990). PCR was performed in a 50 µl reaction system containing 2 µl of DNA template, 2 µl of each 10 µM primer, and 25 µl of 2× Master Mix (Tiangen Biotech Beijing Co. Ltd., Beijing). The PCR protocol was as follows: an initial denaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 2 min, 55°C for 25 s, 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were sent to Invitrogen Biotechnology Co. Ltd. (Beijing, China) for purifying, sequencing, and editing. The other sequence data included in our study were downloaded from GenBank (Table 1).

Phylogenetic analyses

DNA sequences were aligned using Clustal X v1.83 (Thompson et al. 1997), and the alignment was manually adjusted using Se-Al v.2.03a (Rambaut 2000). The aligned dataset was analyzed by maximum parsimony (MP) using PAUP* 4.0b10 (Swofford 2002). MP analysis was conducted using heuristic searches with 1,000 random addition sequence replicates, and the tree bisection reconnection (TBR) branch-swapping algorithm. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions. A bootstrap (BP) analysis was performed with 1,000 replicates, each with 10 random addition sequence replicates, and using TBR branch-swapping. A Bayesian analysis was performed using MrBayes 3.1.2 (Huelsenbeck et al. 2001; Ronquist & Huelsenbeck 2003) with two sets of four chains

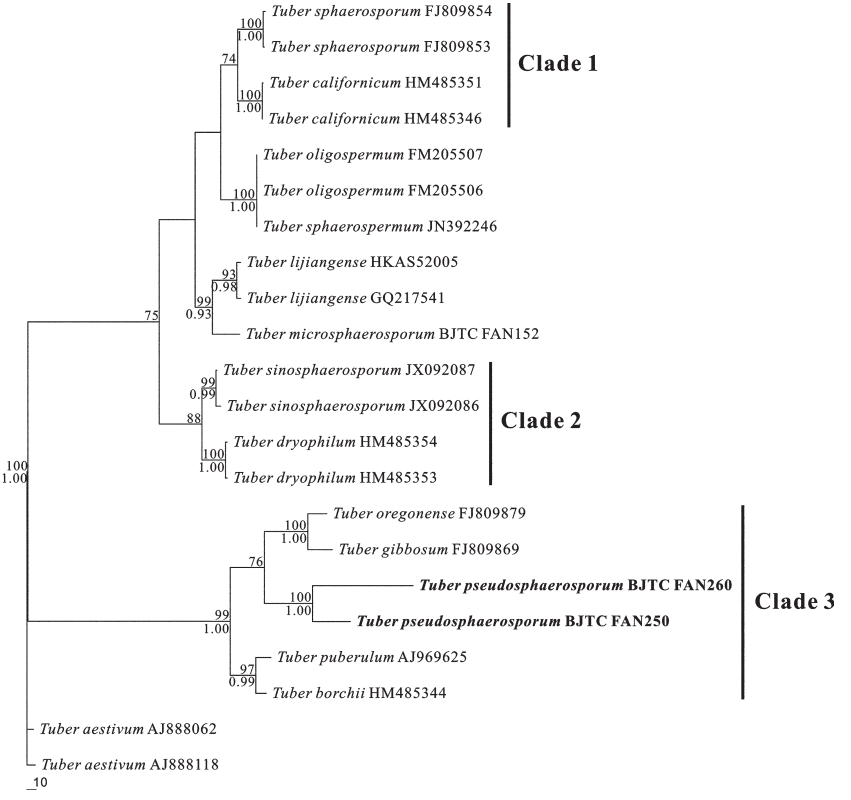

Fig. 1. Phylogeny derived from maximum parsimony analysis of ITS rDNA sequences from some *Tuber* species with globose ascospores. Bootstrap values of >70% are shown above branches. Clades with Bayesian posterior probabilities (PP) estimated at >0.90 are shown under branches. *Tuber aestivum* was used as outgroup. Tree length (TL) = 876 steps, consistency index (CI) = 0.7021, retention index (RI) = 0.7792, homoplasy index (HI) = 0.2979, and rescaled consistency index (RC) = 0.5470.

(one cold and three heated) and the stoprule option in effect, halting the analyses at an average standard deviation of split frequencies of 0.01. The sample frequency was set to 100, and the first 25% of trees were removed as burn-in. Bayesian posterior probabilities (PP) were obtained from the 50% majority rule consensus of the remaining trees. *Tuber aestivum* Vittad. was used as the outgroup.

Results

Molecular phylogeny

Maximum parsimony analysis resulted in one most parsimonious tree (Fig. 1).

Our phylogenetic analysis was based on sequences from 13 *Tuber* species with globose ascospores. It revealed that *T. sphaerosporum*, *T. sinosphaerosporum*, and *T. pseudosphaerosporum* are in separate clades. *Tuber sphaerosporum* clustered with *T. californicum* Harkn. in Clade 1 with moderate support, and *T. sinosphaerosporum* clustered with *T. dryophilum* Tul. & C. Tul. in a well-supported clade (Clade 2 in Fig. 1). A third clade was also strongly supported, and contained *T. pseudosphaerosporum*, *T. oregonense* Trappe et al., *T. gibbosum* Harkn., *T. borchii* Vittad., and *T. puberulum* Berk. & Broome.

Taxonomy

Tuber pseudosphaerosporum L. Fan, **sp. nov.** Fig. 2

MycoBank MB 803932

Differs from other *Tuber* species by its purple-brown gleba and regular globose ascospores with coarse reticulum.

Type: China. Yunnan Province, Huize County, Daibu Town, in the soil under *Pinus armandii*, 11 Nov. 2012, Shuang Feng 017 (**Holotype**, BJTC FAN250).

Etymology: *pseudosphaerosporum* (Lat.), referring to the great similarity with the North American *T. sphaerosporum*.

Ascomata 2–4.5 cm in diam., white, whitish, or whitish-yellow when fresh, subglobose to irregular, mostly much lobed with deep furrows, firm, nearly glabrous. Odor pungent at maturity. Peridium 250–350 μm in thickness, outer layer pseudoparenchymatic, 150–250 μm, composed of subglobose, subangular or irregular cells of 5–25(–40) μm, smaller and larger cells intermixed, cells hyaline with somewhat thickened yellow walls and up to 2.5 μm; toward inner side grading into textura intricata, composed of interwoven hyphae, hyaline, septate, thin-walled, branched, 3–5 μm in diam., occasionally with a few hyphae swollen to 20 μm in diam. Gleba white at first, gradually becoming brown, and finally somewhat dark grey-brown with a purple tint at maturity, marbled with larger whitish meandering veins. Asci 1–3(–4)-spored, hyaline, thin-walled, subglobose to ellipsoid, sessile, 55–85 × 52.5–75 μm. Ascospores brown, regularly globose, reticulate, 25–37.5 μm in diam. excluding ornamentations,

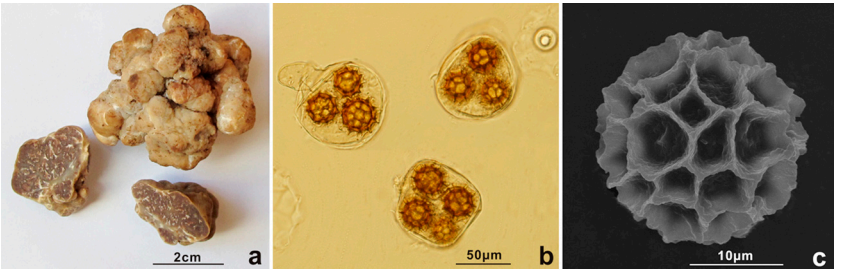

Fig. 2. *Tuber pseudosphaerosporum* (BJTC FAN250, holotype). a. Ascomata; b. Ascospores observed under the light microscope; c. Ascospore observed under SEM.

alveoli large and regular, up to 4–7.5 μm tall and 3–4(–5) alveoli across the spore width.

Additional specimen examined: **China. Yunnan Province**, Huize County, in the soil under *Pinus armandii*, 11 Nov. 2012, Xiao-yong Li 008 (BJTC FAN 260).

Comments—*Tuber pseudosphaerosporum* is morphologically similar to both *T. sinosphaerosporum* and *T. sphaerosporum*. These three species are very easily confused in most of their taxonomic characters (Table 2). Our phylogeny, however, places them in separate clades (Fig. 1), indicating their independent origins. The grey-purple tint in the gleba appears to be unique to the new species and is not observed in *T. sinosphaerosporum* or *T. sphaerosporum* (Gilkey 1954; Fan et al. 2013). In addition, the ascomatal surfaces of both *T. sphaerosporum* and *T. pseudosphaerosporum* are glabrous, while *T. sinosphaerosporum* produces poorly developed and non-specialized septate hyphae-like obtuse hairs on its ascomatal peridium (Fan et al. 2013).

Discussion

Tuber pseudosphaerosporum, *T. sinosphaerosporum*, and *T. sphaerosporum* are very similar morphologically, especially in their ascospore ornamentation — traditionally one of the most important characters used for species differentiation in this genus (Table 2). All three have large-meshed reticulate ascospores (generally 3–4 meshes across the spore width), dark gleba, and pale colored ascomata with nearly glabrous surfaces. Hypha-like hairs can occasionally be found on the peridia of *T. sinosphaerosporum*, but these are usually poorly developed and easily overlooked. It is thus very difficult to separate these species on the basis of morphology. Our phylogenetic analysis however, clearly places the three species in different clades (Fig. 1), indicating that they have evolved in different lineages despite their morphological similarities.

Table 2. Morphological comparison of the three *Tuber* species.

Morphological features	*T. pseudosphaerosporum*	*T. sinosphaerosporum*	*T. sphaerosporum*
Ascocarp color	Whitish	White to whitish	Pale brown
Ascocarp size	2–4.5 cm	1.5–5.5 cm	2.5 cm
Peridium surface	Glabrous	Nearly glabrous	Glabrous
Peridium structure	Irregularly pseudoparenchymatic	Pseudoparenchymatic	Irregularly pseudoparenchymatic
Peridium thickness	150–250 µm	250–300 µm	—
Peridium hairs	Absent	Cylindrical and obtuse	Absent
Gleba	Deep brown with purple tint	Dark brown	Dark brown
Spore number in ascus	1–3(–4)	1–4	—
Spore shape	Regularly globose	Regularly globose	Regularly globose
Spore size	25–37.5 µm	20–45 µm	32–58 µm, including alveoli
Spore ornament	Alveolate	Alveolate	Alveolate
Alveoli number	3–4(–5)	2–4	3–4
Alveoli height	4–7.5 µm	5–7.5 µm	—

Tuber sphaerosporum is known only from North America, and *T. pseudosphaerosporum* and *T. sinosphaerosporum* are known only from southwestern China.

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